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First report of the pantropical species *Diploschistes rampoddensis* from Europe

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Abstract — The lichen species *Diploschistes rampoddensis*, previously known only from a few localities in tropical and subtropical Asia and Oceania, is reported here for the first time in Europe. A detailed description, including macro- and microscopic characters, and comparisons with closely related taxa are also provided. Molecular analyses based on the nrITS were used to confirm this new record for the European lichen biota.

Key words — *Ascomycota,* BAli-phy, disjunct distribution, lichenized-fungi, *Ostropales*

Introduction

The lichen genus *Diploschistes* Norman (*Graphidaceae*, *Ostropales*, *Ascomycota*) currently includes 45 species (Kirk et al. 2008, Pérez-Vargas et al. 2012, Fernández-Brime et al. 2013), distributed mainly in temperate and arid regions of both hemispheres, with only a few species restricted to the tropics (Lumbsch 1989). One of the few species occurring in tropical areas is *D. rampoddensis,* a taxon that was first described by Nylander (1900, as *Urceolaria rampoddensis*) from the locality of Rampodde (Sri Lanka). According to the literature (Nylander 1900, Lumbsch 1993, Pant & Upreti 1993, Martín et al. 2003) and unpublished herbarium specimens, this species appears to be quite rare and has previously been recorded only from a few localities in tropical and subtropical Asia and Oceania.

As part of an ongoing project to assess the lichen diversity of several protected areas of Catalonia (NE Spain), we intensively surveyed the cliffs of Sant Llorenç

de la Muga, a protected area included in the Plan of Sites of Natural Interest (PEIN). Among several *Diploschistes* collections, three samples were initially identified as *D. rampoddensis*, based on their morphological, anatomical, and chemical features. As these specimens were, however, rather scant or inconspicuous, we checked the identification using the nuclear ribosomal internal transcribed spacer (nrITS), recently selected as the universal DNA barcode marker for fungi (Schoch et al. 2012). Our molecular analyses further confirmed that these specimens represent *D. rampoddensis,* which extends its distributional range to Europe.

Materials & methods

Morphological analysis

The morphological study was carried out on fresh specimens collected from the cliffs of Sant Llorenç de la Muga and on herbarium material of *Diploschistes rampoddensis* lodged and loaned from UPS, as well as on other taxa of *Diploschistes* used for comparison. Morphological characters were examined with an Olympus VMZ dissecting microscope (40×). Anatomical characters were studied on hand-cut sections mounted in water (40×) or oil immersion (100×) using an Olympus CH-2 light microscope. Vouchers of new specimens were conserved in CeDocBiV , Universitat de Barcelona, Spain (BCN), Institute of Botany, Academy of Sciences, Czech Republic (PRA), and the first author's private herbarium (Hb. Fdez.-Brime, SFB).

Selected specimens were photographed using a Pixera Pro150 ES camera mounted on an Olympus SZ60 stereomicroscope. For each selected specimen, multiple images in different focal planes were taken and subsequently assembled into a final unique picture using the software Helicon Focus 5.2.

Spot tests were performed using standard lichenological chemical reagents (K, C) along with thin-layer chromatography (TLC) following the protocols of Orange et al. (2001).

Molecular analysis

Genomic DNA was obtained from small pieces of thallus and apothecia sampled from fresh specimens using a phenol-chloroform-isoamyl alcohol extraction protocol based on Lee et al. (1988). Approximately 560 bp including the complete nrITS region was amplified using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). PCR mixtures were prepared as in Gueidan et al. (2007) and conditions for thermocycling followed Fernández-Brime et al. (2013). The nrITS region was sequenced as detailed in Martín & Winka (2000), using the same amplification primers.

We aligned the nrITS of the three specimens tentatively identified as *Diploschistes rampoddensis* with the only nrITS sequence of *D. rampoddensis* available from GenBank, together with other sequences of closely related representatives of *Diploschistes* subg. *Diploschistes* (Fernández-Brime et al. 2013). *Diploschistes actinostomus* (Ach.) Zahlbr. and *D. candidissimus* (Kremp.) Zahlbr., from *Diploschistes* subg. *Limborina* Fdez.-Brime et al. (Fernández-Brime et al. 2013) were selected as outgroups to root the phylogeny. A total of 17 sequences were used for the phylogenetic analyses (Table 1). All newly

Table 1. Specimens and sequences of *Diploschistes* used in the present phylogenetic analysis.

Species	Voucher	Origin	GenBank*
D. actinostomus	BCC-Lich 13394	Spain, Catalonia	AF229194
D. candidissimus	ESS 20699	Australia, South Australia	AJ458281
D. cinereocaesius	ESS 9364	Venezuela, Mérida	AJ458282
	DUKE 0047509	Costa Rica, San José	HQ650715
	Hb. Palice, Palice 4471	Ecuador	**KJ542542**
D. rampoddensis	Hb. Lumbsch, Aptroot 39679	Papua New Guinea	AJ458286
	BCC-Lich 18009	Spain, Catalonia	KC166992
	BCC-Lich 18011	Spain, Catalonia	KC166993
	BCC-Lich 18008	Spain, Catalonia	**KJ542543**
D. scruposus	BCN-Lich 19326	Spain, Catalonia	KC167067
	Hb. Fdez.-Brime, SFB 59	Spain, Catalonia	**KJ542545**
	Hb. Fdez.-Brime, SFB 103	Spain, Catalonia	**KJ542546**
	ESS 21508	Germany, Rheinland-Pfalz	AJ458287
D. scruposus "*interpediens*"	BCN-Lich 19319	Spain, Catalonia	KC166995
	BCN-Lich 19355	Spain, Catalonia	KC166999
	BCN-Lich 19322	France, Languedoc-Roussillon	KC167003
	Hb. Fdez.-Brime, SFB 104	Spain, Catalonia	**KJ542544**

* Newly obtained sequences shown in bold.

generated DNA sequences are now deposited in GenBank (KJ542542–KJ542546) and the alignment is available in TreeBASE (http://www.treebase.org; ID number 15496).

Sequences were manually aligned with Mesquite 2.75 (Maddison & Maddison 2011). Terminal primers and partial nuSSU and nuLSU were excluded from the alignment. Phylogenetic relationships were inferred using weighted maximum parsimony (wMP) and Bayesian methods (B). The wMP analyses were carried out in PAUP* v.4.0b10 (Swofford 2002), with gaps treated as a fifth character state in unambiguously aligned regions. These regions were subjected to symmetric step matrices (i.e., ITS1, 5.8S, and ITS2 being treated separately), using STMatrix v.3.0 (F. Lutzoni & S. Zoller, Dept. of Biology, Duke University), as outlined in Gaya et al. (2003). Ambiguously aligned regions were removed from the wMP searches and recoded and incorporated into the analyses using INAASE v.2.3b (Lutzoni et al. 2000), as in Gaya et al. (2008). All wMP analyses were performed using heuristic searches with 1000 random addition sequences (RAS), the tree bisection-reconstruction (TBR) algorithm, MULTREES in effect, and collapsing branches with maximum branch length equal to zero. Branch support (BS) was assessed with 1000 bootstrap replicates (Felsenstein 1985) with full heuristic searches, and 2 RAS per bootstrap replicate. In all BS analyses, the same parameters as in the original MP search were used, and constant sites were excluded from all analyses. The Bayesian analyses (B) were done using BAli-Phy 2.1.1 (Suchard & Redelings 2006), a software that simultaneously accounts for alignment uncertainty and phylogeny, allowing the inclusion of the whole alignment. In this case, the nrITS dataset was divided into three partitions (ITS1, 5.8S, and ITS2) as suggested in Gaya et

al. (2011). The analyses were run under the following settings: ITS1 and ITS2 sharing the same model of evolution (HKY+G), indel model (RS07; Redelings & Suchard 2007) and branch length estimation, while the EQU model and separate branch length estimation were applied to the partition 5.8S, which was fixed because the alignment was unambiguous. We ran 6 independent Markov chains of 50,000 iterations each, sampling the Markov chains every 10 iterations. After discarding the first 1250 samples of each run (25%), we computed the majority rule consensus with the remaining trees (22,500).

Results & discussion

Molecular phylogenetics

The final nrITS alignment included 17 taxa and comprised 594 characters. We excluded from the wMP analyses 557 characters, of which 407 were constant sites and 150 corresponded to 19 ambiguously aligned regions. The characters from the ambiguously aligned regions were included as 19 INAASE recoded characters. From the 56 variable characters analyzed by wMP, 40 were parsimony-informative. We included the whole alignment for the B analyses.

The wMP search yielded six most parsimonious trees of 159.27 steps, which were found in one island hit 991 times out of 1000 RAS. The strict consensus tree (Fig. 1) shows 13 resolved internodes, of which nine were significantly supported (BS ≥70%). The 50% majority rule consensus tree from the B analyses showed slightly less resolution, with nine resolved internodes, but, as with wMP, nine of these were significantly supported (PP ≥0.95).

The three European specimens putatively representing *Diploschistes rampoddensis* clustered with the only sequence of *D. rampoddensis* from GenBank and formed a well-supported clade (BS = 100%; PP = 1). This clade was sister to *D. cinereocaesius* (Sw.) Vain., which provided support for this sister relationship for the first time. In contrast, specimens of other taxa [e.g., *Diploschistes scruposus* (Schreb.) Norman, *D. scruposus* "morphotypus *interpediens*")], even if morphologically similar and collected from the same locality of Sant Llorenç de la Muga (specimens marked with an asterisk in Fig. 1), clustered separately from *D. rampoddensis.*

Taxonomy

Diploschistes rampoddensis (Nyl.) Zahlbr., Cat. Lich. Univers. 2: 665, 1924. Fig. 2A

Thallus saxicolous, crustose, yellowish grey to greenish yellow, without pruina but with a crystalliferous surface, 0.2–0.9 µm thick, friable (easily broken), from verrucose-areolate to mostly reduced into conglutinated granules; epinecral layer 24–31 µm thick, without a well-developed cortex; photobiont trebouxioid.

Apothecia urceolate, 0.2–1.3(–1.7) mm in diam., never subdivided, with a deeply concave black disc with white pruina. Excipulum black, 60–100

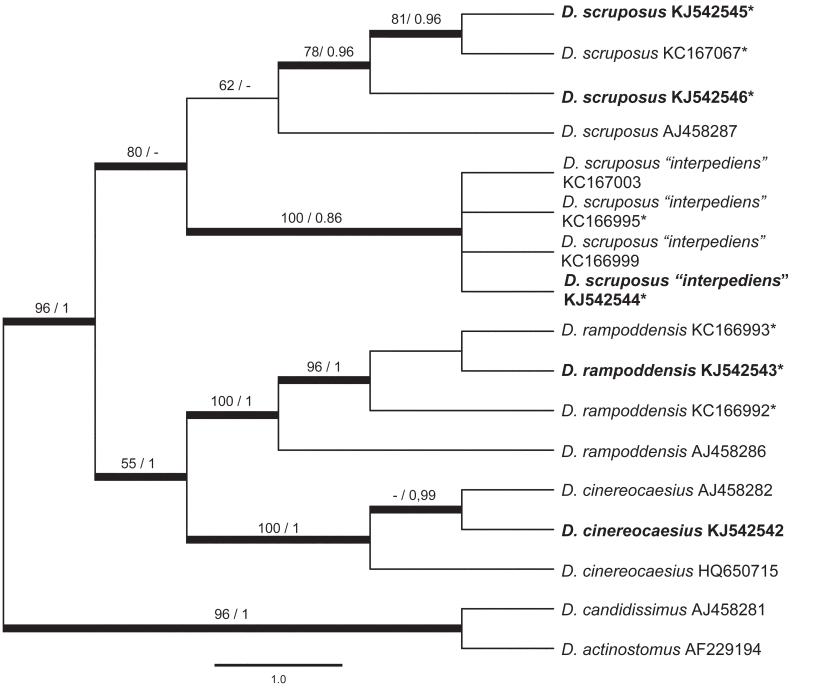

Fig. 1. Phylogenetic relationships of sequences of *Diploschistes* species summarized by a strict consensus tree of six equally most parsimonious trees, as revealed by the wMP analyses of the nrITS dataset, including 19 INAASE recoded characters. Bootstrap support ≥50% (BP; left) and posterior probabilities ≥0.5 (PP; right) are shown above branches. Internodes with BP values ≥70% or PP ≥0.95 are highlighted by thicker lines. Asterisk-marked specimens collected in the same locality of Sant Llorenç de la Muga (Catalonia, Spain). Newly generated sequences are presented in bold font.

(–130) µm thick, lateral paraphyses present. Hymenium hyaline to pale brown, (78–)98–128(–134) µm high. Asci clavate, unitunicate, non-amyloid walls, (6–)8 spores per ascus, 100–170 × 15–30 µm. Ascospores ellipsoid, pale to dark brown, non-amyloid, (19.5–)21– 26.5(–29) × (8.5–)9–12(–13.5) µm (n = 59), with 4–7(–8) transverse septa, and 1–2 longitudinal septa.

Chemistry — Spot test reactions of the thallus K– or K+ pale yellow, C+ and KC+ pink (brief reaction). TLC: lecanoric acid as major compound, compounds in traces were not found.

Distribution & habitat — Since *Diploschistes rampoddensis* was first described by Nylander (1900) from material collected in Rampodde, Sri

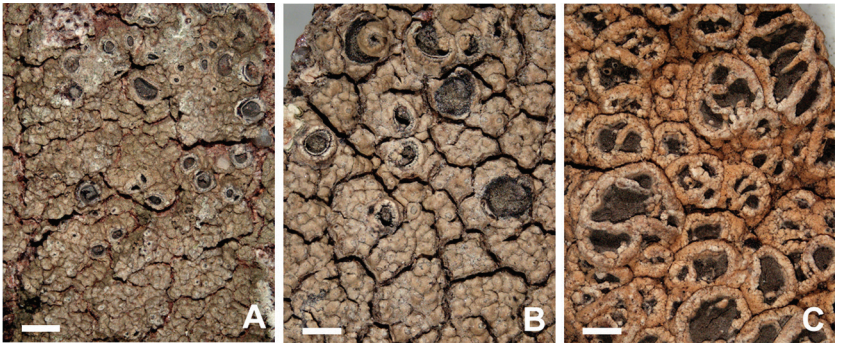

Fig. 2. A. *Diploschistes rampoddensis* (BCN-lich. 18011): habit. B. *D. scruposus* "*interpediens*" (BCN-lich. 14404): habit. C. *D. cinereocaesius* (BCN-lich. 18010): habit. Scale bars = 1 mm.

Lanka, it has been recorded from a few Asian and Australasian localities: China (see examined specimens), India and Nepal (Pant & Upreti 1993), Indonesia (Lumbsch 1993), and Papua New Guinea (Martín et al. 2003). The lichen usually occurs in lowlands and only rarely appears at high elevations of the intertropical region (Lumbsch 1993). Our study significantly extends its distribution to Europe, where it is reported only from a single locality, Sant Llorenç de la Muga (Catalonia, Spain). This area's warm humid Mediterranean climate (with relatively high temperatures and humidity) has allowed the establishment of a rich foliicolous lichen flora (Llop & Gómez-Bolea 2006), predominantly Atlantic bryophyte species such as *Leucobryum juniperoideum* (Brid.) C. Müll. (Brugués et al. 1974), and the pteridophyte *Pellaea calomelanos* (Sw.) Link earlier known from South Africa, northern India, and Macaronesia (Terradas & Brugués 1973). The existence of these Atlantic and paleotropical elements in the study area would agree with the presence of *Diploschistes rampoddensis* outside its known pantropical distribution.

The European *D. rampoddensis* samples are saxicolous and grow on Garumnian red beds (HCl–). Pant & Upreti (1993), however, reported the species colonizing sandstones from India.

Specimens examined — **CHINA. Yunnan.** Simao Distr. Mojiang Co., Mojiang (km-post 350 from Kunming), along the main road, 23°26′N 101°41′E, 1200 m, on hard soil, 12 Sep. 1987, Moberg & Santesson 7702 (UPS L-11539); along the road E of the river Bavian Jiang (km-post 433 from Kunming), 23°16′N 101°16′E, 1100 m, on wayside rocks, 13 Sep. 1987, Moberg & Santesson 31970 (UPS L-11540). **SPAIN. Catalonia.** Sant Llorenç de la Muga, path to Can Gener, UTM 31TDG8388, 150 m, Garumnian red beds, 9 Feb. 2007, Llimona & Hladun (BCN-lich. 18011); cliffs by the river la Muga, near an old mine, UTM 31TDG8188, 280 m, Garumnian red beds 45° S-SW, 2 Mar. 2007, Llimona, Hladun & Muñiz (BCN-lich. 18008, GenBank KJ542543; BCN-lich. 18009).

Remarks —*Diploschistes rampoddensis* resembles octosporate specimens of *D. scruposus* (Fig. 2B; *D. scruposus* "morphotypus *interpediens*", hereafter abbreviated to *D. scruposus* "*interpediens*"), which also have (6–)8 ascospores per ascus and a similar ascospore size [(21–)23.5–27 × (9.5–)10–13(–13.5) µm]. However, a detailed morphological comparison reveals that *D. rampoddensis* has a thinner and more delicate thallus than *D. scruposus* "*interpediens.*" Their chemistry also differs: *D. rampoddensis* contains only lecanoric acid (major), while *D. scruposus* "*interpediens*" contains diploschistesic acid (minor) and orsellinic acid (minor), in addition to lecanoric acid (major) (Barbero 1998). Morphological and chemical differences between these two taxa are nonetheless really subtle, and the utilization of the nrITS was crucial in confirming the identity of the European specimens of *D. rampoddensis.*

Within *Diploschistes,* there are two other pantropically distributed species (*D. cinereocaesius* and *D. hypoleucus* Zahlbr.), which resemble *D. rampoddensis* in having a yellowish thallus, octosporous asci, and a rather similar ascospore size. These two species, however, are terricolous, have bigger apothecia that are usually secondarily subdivided (see *D. cinereocaesius,* Fig. 2C), and have different chemistry: *D. cinereocaesius* contains lecanoric acid (major) and diploschistesic acid (minor), while *D. hypoleucus* contains gyrophoric acid as a major compound. That *Diploschistes rampoddensis* and *D. cinereocaesius* appear as sister taxa in our phylogeny suggests a biogeographic pattern.

Additional specimens examined — *Diploschistes cinereocaesius*. **COLOMBIA. Urrao.** Páramo de Frontino, 6°25′N 76°05′W, 3500 m, 30 June 1985, Churchill, Sastre-De Jesús & Escobar (DUKE 0144448). **ECUADOR. Imbabura.** Laguna Cuicocha - NE edge, ca 9km W of town Cotacachi, 00°18′43″ N, 78°21′15″ W, on soil at trail-cutting, 3300–3350 m, 8 Oct. 2000, Palice 4471 & Soldán (PRA, GenBank KJ542542) **PERU. Huaraz.** Route to Laguna Churup, 9°29′S 77°26′W, 4070 m, 25 Aug. 2010, Rothfels 4000 & Zylinski (BCN-lich. 18010).

Diploschistes scruposus. **SPAIN. Catalonia**. Girona, Sant Llorenç de la Muga, cliffs by the river la Muga, near an old mine, UTM 31TDG8188, 280 m, Garumnian red beds 45° S-SW, 2 Mar. 2007, Llimona, Hladun & Muñiz (Hb. Fdez.-Brime, SFB 59, GenBank KJ542545; BCN-Lich 19326); cliffs by the river la Muga, UTM 31TDG8188, 240 m, granitic outcrops 40° S-SW, 3 June 2009, Fernández-Brime (Hb. Fdez.-Brime, SFB 103, GenBank KJ542546).

D. scruposus "*interpediens*". **FRANCE**. **Pyrenées Orientales**: Albera, road from Maurellans to Rinoguers, 31TDH85351, 300 m, granitic outcrops near the road, 13 June 2009, Llimona (BCN-Lich 19322). **SPAIN. Catalonia**. Barcelona, Òrrius, turó de Céllecs, 31T DG4400, 530 m, granitic outcrops, 23 Jan. 2009, Llimona & Fernández-Brime (BCN-Lich 19355). Girona, Sant Llorenç de la Muga, path to Can Gener, 31T DG8387, 140-150 m, Garumnian red beds, 9 Feb. 2007, Llimona & Hladun (BCN-lich. 19319); cliffs by the river la Muga, UTM 31TDG8188, 260 m, granitic outcrops 60° E, 3 June 2009, Fernández-Brime (Hb. Fdez.-Brime, SFB 104, GenBank KJ542544). **La Rioja**. Anguiano, electric power station, 30T WM1878, 670 m, *Quercus ilex* forest, 8 Sep. 2004, Hladun & Muñiz (BCN-Lich 14404).

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